Title
Expression of human adenosine deaminase in nonhuman primates after retrovirus-mediated gene transfer.

Journal
The Journal of experimental medicine, 166(1)

ISSN
0022-1007

Authors
Kantoff, PW
Gillio, AP
McLachlin, JR
et al.

Publication Date
1987-07-01

DOI
10.1084/jem.166.1.219

Peer reviewed

EXPRESSION OF HUMAN ADENOSINE DEAMINASE IN NONHUMAN PRIMATES AFTER RETROVIRUS-MEDIATED GENE TRANSFER

By PHILIP W. KANTOFF,* ALFRED P. GILLIO,‖ JEANNE R. McLACHLIN,* CLAUDIO BORDIGNON,‖ MARTIN A. EGLITIS,* NANCY A. KERNAN,‖ ROBERT C. MOEN,* DONALD B. KOHN,§ SHEAU-FUNG YU,‖ EVELYN KARSON,* STEFAN KARLSSON,‡ JAMES A. ZWIEBEL,* ELI GILBOA,‖ R. MICHAEL BLAESE,§ ARTHUR NIENHUIS,‡ RICHARD J. O'REILLY,‖ AND W. FRENCH ANDERSON*

*From the *Laboratory of Molecular Hematology and the ‡Clinical Hematology Branch, National Heart, Lung, and Blood Institute; the §Metabolism Branch, National Cancer Institute; National Institutes of Health, Bethesda, Maryland 20892; and the ‖Memorial Sloan-Kettering Cancer Center, New York 10021*

The development of retroviral vectors capable of efficient insertion of genes into mammalian hematopoietic cells (1–7) has generated interest in gene therapy as a potential approach for the treatment of lethal genetic disease (8, 9). The cloning of a cDNA for the human adenosine deaminase enzyme (h-ADA)[1] (10–12) has made the ADA deficient form of severe combined immunodeficiency (SCID) a potential candidate for such therapy. We recently described (13) a retroviral vector called SAX that contains the h-ADA cDNA (Fig. 1). Using this vector, the h-ADA gene was transferred into ADA-deficient human T cells that had been previously immortalized with human T cell leukemia virus (HTLV-1). After gene transfer, the T cells produced the ADA enzyme from the new gene at levels similar to those seen in normal cells. This level of expression was sufficient to make the treated cells resistant to levels of deoxyadenosine that are toxic to untreated ADA-deficient T cells in vitro (13). Therefore, the SAX retroviral vector appears to function efficiently in T cells cultured in vitro from ADA-deficient patients.

Several groups have attempted to establish in vivo models of h-ADA gene transfer in the mouse. Considerable success has been achieved in transferring the h-ADA gene into murine hematopoietic cells (14), and recently expression

E. Gilboa is supported by a grant from the March of Dimes 1-1047. A. Gillio, C. Bordignon, N. A. Kernan, and R. J. O'Reilly are supported in part by U.S. Public Health Service grant CA-33050 and CA-08748 awarded by the National Cancer Institute, as well as contributions from the Zelda Weintraub Cancer Foundation, Robert J. Kleberg and Helen C. Kleberg Foundation, the Yankee-Frito Lay Challenge Foundation, Lila Acheson Dewitt Wallace Foundation, the Vincent Astor Chair for Clinical Research, the Andrew Gaffney Foundation, and the Panwy Foundation. A. Gillio is supported in part by the Toys-R-Us Foundation. C. Bordignon is a Leukemia Society of America Special Fellow. N. A. Kernan is supported in part by a Clinical Scholars Award from the Norma and Rosetta Winston Foundation.

[1] *Abbreviations used in this paper:* ADA, adenosine deaminase; FPLC, fast protein liquid chromatography; NPT, neomycin phosphotransferase; SV40, simian virus 40.

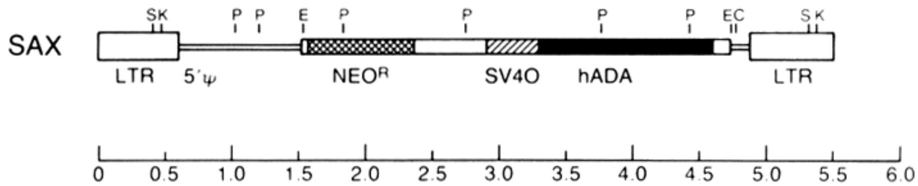

FIGURE 1. Map of SAX vector. The following regions are indicated: 0–1.5 and 4.7–5.5 kb, Moloney murine leukemia virus sequences; 1.5–2.9 and 4.6–4.7 kb, neomycin-resistance gene (*neo*^{r}) from Tn 5 transposon (the hatched area is the coding sequence); 2.9–3.3 kb, Kpn I–Hind III fragment of the SV40 early promoter; 3.3–4.6 kb, human ADA cDNA (h-ADA, black box); LTR, viral long terminal repeat; 5′,5′ donor splice site; φ, viral packaging signal. Restriction sites: *S*, Sac I; *K*, Kpn I; *P*, Pst I; *E*, Eco RI; *C*, Cla I. Scale (kb) at bottom.

of this gene has been observed in spleen foci (15), but not yet in the fully reconstituted animal. To test the feasibility of a bone marrow transplant/gene transfer approach in a large animal model more analogous to man, we have developed a primate autologous transplantation model for the study of retrovirus-mediated ADA gene transfer into bone marrow cells. This report provides evidence for a low level of transfer and expression of the h-ADA gene in circulating hematopoietic cells in cynomolgus monkeys for short periods of time.

Materials and Methods

Animals and Cell Lines. The animals used in these studies were cynomolgus macaque monkeys obtained from Hazelton Research Animals. NIH 3T3 cells were kindly provided by Dr. Sandra Ruscetti (NCI, NIH) and the PG-4 cell line (used as a helper virus indicator for S^+L^- assays) by Dr. Robert Bassin (NCI, NIH).

Vector Construction. The construction of the retroviral vector SAX (Fig. 1) and the generation of the virus producer cell line S3A have been described previously (13). Briefly, SAX was made by inserting simian virus 40 (SV40)-promoted human ADA cDNA into the previously described (5, 6, 16) parental vector, N2. A fusion gene was created between the SV40 promoter and the ADA structural gene by placing the 400 bp Kpn I–Hind III fragment containing the enhancer and promoter elements of the SV40 early region immediately upstream of a 1,300 bp sequence containing the full-length ADA cDNA (10) (Eco RI–Acc I fragment of clone ADA 211).

Bone Marrow Processing and Transplantation. Marrow (~40–60 ml) was harvested from the long bones of the animal before irradiation. Mononuclear cells were isolated by 3% gelatin sedimentation and Ficoll/Hypaque gradient separation. These cells were then incubated with vector-producing cells or vector-containing supernatants at 37°C in the presence of 8 μg/ml polybrene (Aldrich Chemical Co., Milwaukee, WI) as described in the text. The cynomolgus primates received a dose of 1,000 rad total body irradiation (^{60}Co at 10 rad/min) immediately followed by infusion of their autologous bone marrow infected with the SAX vector. This dose of irradiation produces permanent aplasia in unreconstituted animals in this primate model.

Hematopoietic Cell Culture. The CFU-C assay for myeloid progenitors has been described (17, 18). The assay medium was McCoy's supplemented with 20% prescreened heat-inactivated FCS (Hyclone Laboratories, Logan, UT), 10% giant cell tumor–conditioned medium (Gibco, Grand Island, NY) as a source of granulocyte/macrophage colony-stimulating factor (GM-CSF), and 0.6% agarose (Gibco). CFU-C were allowed to grow at 37°C and were counted on day 14–17 after plating.

DNA Southern Blotting. High-molecular-weight DNA was prepared by the method of Gross-Bellard (19) and was digested with Kpn I restriction endonuclease (New England Biolabs, Beverly, MA), electrophoresed on a 1.0% agarose gel (FMC Bioproducts, Rockland ME), and transferred to nitrocellulose (Schleicher and Schuell, Keene, NH) by the method of Southern (20). The blot was hybridized with a ^{32}P-labelled *neo*^{r} (neomycin resistance) gene probe (sp act $>10^8$ dpm/μg).

Adenosine Deaminase Assay. The separation of primate from human adenosine deaminase activity using fast protein liquid chromatography (FPLC) has been described in detail elsewhere (21). Briefly, proteins were fractionated on a Pharmacia Mono Q column (HR 5/5) using a linear gradient of 0.05–0.5 M KCl, 20 mM Tris-HCl (pH 7.5). ADA activity in column fractions was determined by measuring the conversion of [^{14}C]adenosine (Amersham Corp., Arlington Heights, IL) to [^{14}C]inosine followed by thin-layer chromatographic separation using 0.1 M Na_2HPO_4 (pH 6.8), saturated ammonium sulfate and *n*-propylalcohol (100:60:2), according to the method of Soberman and Karnovsky (22).

Neomycin Phosphotransferase (NPT) Assay. Cell lysates were made from nucleated cells derived from Ficoll-Hypaque separation. The lysates were electrophoresed for 15 h at 50 V on a nondenaturing polyacrylamide (Pharmacia Fine Chemicals, Piscataway, NJ) gel system and transfer of ^{32}P from γ[^{32}P]ATP (Amersham) to a Kanamycin (Sigma Chemical Co., St. Louis, MO) substrate was achieved using the method of Riess et al. (23) to detect NPT activity.

In Situ Hybridization. Marrow or peripheral mononuclear hematopoietic cells, after Ficoll-Hypaque separation, were adjusted to 2×10^5 cells/ml in Dulbecco's Minimum Essential Medium (Biofluids, Rockville, MD) containing 10% FCS (Gibco). Cells were transferred onto poly-L-lysine (Miles Laboratories, Naperville, IL)-coated slides by cytocentrifugation and fixed with freshly made 4% vol/vol paraformaldehyde (Sigma Chemical Co.) in PBS (Biofluids) containing 5 mM $MgCl_2$ (Sigma Chemical Co.) for 15 min at room temperature. The slides were stored at 4°C in 70% vol/vol ethanol/water. The methods of hybridization, washing, and exposure of film emulsion were those of Singer et al. (24). The probe used was an ^{35}S-labelled *neo*^{r} probe.

T Cell Cloning. T cell cloning was performed using the methods previously described by Kernan et al. (25).

Results

Infection of Primate Hematopoietic Cells In Vitro. Initial studies in our primate model were undertaken to establish whether and to what degree primate hematopoietic cells could be infected by the SAX vector and express the vector-containing *neo*^{r} (NPT) and h-ADA genes. Conditions were established for in vitro analysis of infection of hematopoietic progenitor cells, based on the resistance of cells expressing the *neo*^{r} gene to the toxic neomycin analogue G418. As shown in Fig. 2, the growth of normal cynomolgus marrow CFU-C (curve *A*) is completely suppressed at a G418 concentration of 0.29 mg/ml and above.

Two protocols for infecting bone marrow progenitor cells in vitro were used. In the first, Ficoll/Hypaque-separated bone marrow mononuclear cells were cocultured for various periods of time with the SAX vector–producing NIH 3T3 cell clone S3A. This producer cell line, derived from the PA12 amphotropic packaging cell line (26), generates infectious SAX virus at concentrations as high as 6.0×10^6 *neo*^{r} CFU/ml. Cocultivation of cynomolgus monkey bone marrow mononuclear cells with an S3A monolayer for periods ranging from 4 to 24 h conferred resistance to 1.7 mg/ml G418 in a maximum of 7% (at 24 h) of the CFU-C detected on day 14 (Fig. 2, curve *B*). In the second protocol, bone marrow cells were exposed to virus-containing supernatants derived from the S3A producer cells, using ratios (SAX virus/bone marrow mononuclear cells) of between 5 and 10 to 1. After 2 h of exposure followed by extensive washing with PBS, >10% of day 7 CFU-C (data not shown) and 28% of the day 14 CFU-C (Fig. 2, curve *C*) detected were resistant to toxic concentrations of G418.

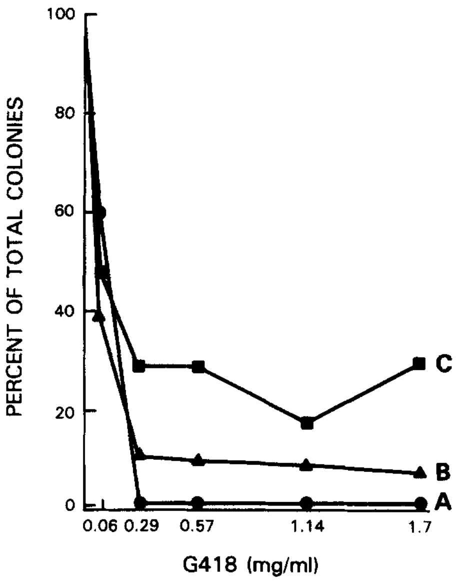

FIGURE 2. Susceptibility of cynomolgus primate myeloid progenitors (day 14 CFU-C) to increasing concentrations of the neomycin analogue G418. *A*: survival of control noninfected colonies. *B*: survival after a 24-h cocultivation of bone marrow cells on a monolayer of SAX-producing NIH 3T3 cells. *C*: survival of marrow cells from animal 57 after a 2-h incubation with a cell-free supernatant containing the SAX vector at a titer of 2×10^6 neo^r CFU/ml. Triplicates of 10^5 bone marrow mononuclear cells per plate were used for each experimental point. The total number of colonies in the absence of G418 was 36–58 per plate. The indicated concentrations of G418 (active; dry weight is twice as high) were added to the culture system immediately before plating. Colonies were counted on day 14.

In Vivo Studies. The in vitro results outlined above were subsequently supported by the results of autologous bone marrow transplants of SAX vector–infected bone marrow cells administered to cynomolgus monkeys. For these experiments, 40–60 ml of heparinized bone marrow was collected from the animal. After gel sedimentation and Ficoll/Hypaque separation to remove red blood cells, the washed bone marrow cells were infected with the SAX vector, either by cocultivation with S3A producer cells for 18–24 h or by incubation with S3A-derived cell-free supernatants for 2 h. During the separation and infection of marrow cells, the animals underwent total body irradiation, receiving a midline dose of 1,000 rad. There is no animal model for ADA deficiency and, therefore, the monkeys required lethal irradiation to ablate their remaining marrow and make space for the treated cells. The SAX-infected bone marrow cells were then infused back into the same animal. After transplantation, the animals were maintained in filtered air reverse isolation, and received transfusions of blood products, antibiotics, and intravenous nutritional support by an indwelling catheter in the superior vena cava until they achieved normal hematopoietic function and reconstitution of all blood cell lines. At intervals after transplantation, blood and bone marrow samples or tissues obtained at autopsy were analyzed for vector DNA and for both h-ADA and NPT activities. The results of the first six bone marrow/gene transplants are summarized in Table I.

Cocultivation Protocol. The first two monkeys received bone marrow that had been cocultured with a monolayer of S3A producer cells (Table I). One of these animals (855) died of systemic bacterial infection 30 d after transplant without evidence of recovery of normal hematopoietic function, and was not studied. The other animal (10) experienced a rapid recovery of his white blood cells (achieving an absolute neutrophil count of 1,000 cells/mm^3 by day 20), but never achieved a normal platelet count throughout the posttransplant period (Fig. 3). Animal 10 was sacrificed at day 69 and the tissues were analyzed for evidence of

TABLE I

Summary of Primate Autologous Bone Marrow Transplantation/Gene Transfer Experiments

Primate	Mode of infection*	Exposure time	Cells infected	Cells infused	NPT‡	h-ADA‡	Endogenous ADA§	Outcome
		h	$\times$ *10⁷/kg*	$\times$ *10⁷/kg*			%	
10	C	24	7.4	1.1	+	+	<0.01	Sacrificed day 69
855	C	24	2.0	1.0	ND	ND	ND	Died of sepsis day 30
56	S	2	11.0	11.0	+	+	0.2	A & W‖ >1 yr
57	S	2	20.0	20.0	+	+	0.5	A & W >1 yr
77	S	2	29.0	29.0	–	–	0	A & W >9 mo
78	S	2	74.0	74.0	–	+	0.05	A & W >9 mo

* Method of infection (C, coculture; S, supernatant).

‡ Assayed in the posttransplant period.

§ Total h-ADA activity (at its maximum; see Fig. 6) expressed as percentage of total endogenous primate ADA activity. The conversion of [^{14}C]adenosine to [^{14}C]inosine reached 66% in fraction 3 of the (FPLC) fast protein liquid chromatography column shown in Fig. 5, which is the region where h-ADA elutes. The endogenous monkey ADA activity is 100% in a number of fractions (17–22). These values were obtained from 2-h incubations at 37°C. To determine the ratio of human to monkey activity, each fraction in the human and monkey regions was reassayed at 1, 3, 5, 10, 30, and 60 min. The amount of ADA activity in each fraction could then be quantitated. The percentage value shown represents 100 × (total h-ADA activity)/(total monkey endogenous ADA activity).

‖ A & W, alive and well.

gene transfer. A ^{32}P-labelled *neo*^{r} gene probe was used for Southern blot analysis of PBMC, bone marrow, and spleen DNA digested with Kpn I restriction endonuclease. As shown in Fig. 4, a 4.9 kb Kpn I band, representing the known unit length fragment characteristic of the intact SAX vector (see Fig. 1), was detected in blood and marrow but not spleen. This band was also present in the plasmid control lane, but was absent in the lane containing DNA from the PBMC of a noninfected monkey. Analysis of the blot suggests that the equivalent of a single-copy of the vector sequence was present in 5–10% of blood and marrow cells, because these bands are less than one-tenth the intensity of the plasmid control that contained a quantity of DNA equivalent to approximately one copy per cell. Similar results were obtained probing the same blot with a ^{32}P-labelled h-ADA gene. However, in addition to hybridization of sequences in the bone marrow and peripheral blood, a light band was detected at 4.9 kb in the spleen lane (data not shown).

PBMC obtained from animal 10 on day 69 were also analyzed for h-ADA and NPT activities. Fractionation of cell proteins by FPLC MonoQ ion-exchange chromatography effectively separates human from primate ADA as assessed by starch gel analysis of species specific ADA isozymes (21). In animal 10, 1% conversion of ^{14}C-labelled adenosine to [^{14}C]inosine can be detected in the fractions in which human but not monkey ADA elutes. The human ADA activity detected was calculated to be <0.01% of the endogenous primate ADA activity (see legend, Table I). NPT activity (which elutes from the column later, see Fig. 5) was also detected in the PBMC at low levels at the same time.

Supernatant Infection Protocol. In the first two transplants there was poor recovery of bone marrow cells from the tissue culture dishes after the 24-h

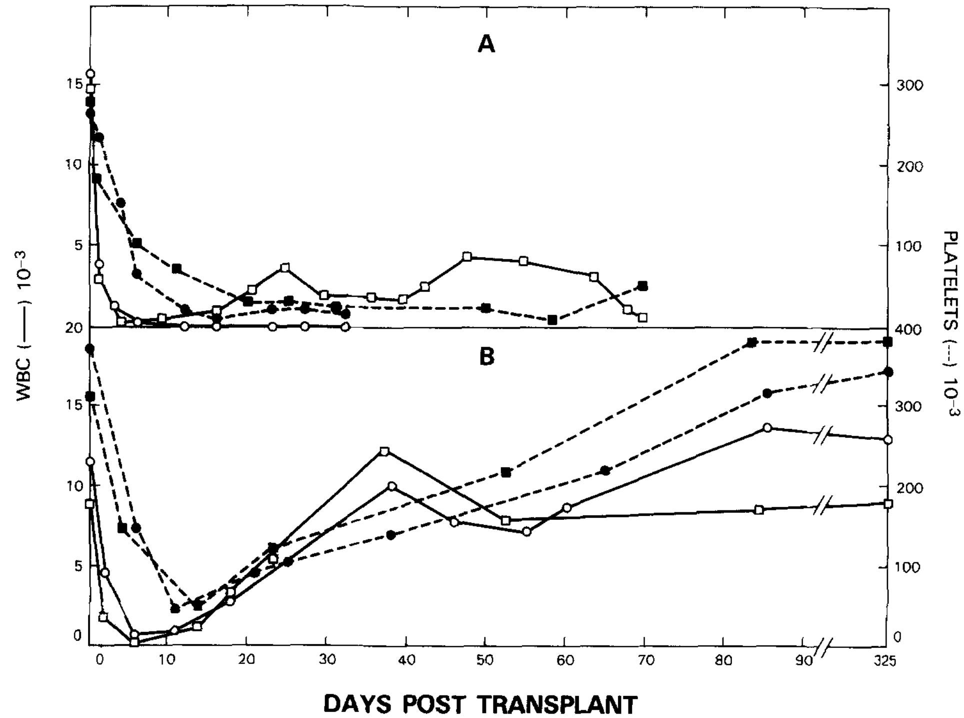

FIGURE 3. Hematopoietic reconstitution of primates after transplantation. *A*: the hematopoietic reconstitution of white blood cells (*open symbols*) and platelets (*closed symbols*) of primates 10 (*squares*) and 855 (*circles*), whose marrow cells were cocultivated for 24 h on a monolayer of SAX-producing NIH 3T3 cells. After coculture, the nonadherent cells were washed with PBS and infused back into the animal. *B*: the hematopoietic reconstitution of primates 56 (*squares*) and 57 (*circles*) that received autologous bone marrow cells infected during a 2-h incubation with a cell-free supernatant containing SAX vector at a titer of 2×10^6 *neo*^{r} CFU/ml. Two additional primates described in this paper were also transplanted with a similar protocol (Table I, primates 77 and 78); their pattern of reconstitution was similar to those of primates 56 and 57.

cocultivation period (Table I). In an attempt to increase the number of cells recovered after transfer manipulations and to eliminate the possibility of S3A cell contamination, the subsequent two vector transfers (animals 56 and 57) were performed by incubating bone marrow cells with a filtered (0.22 μm) supernatant collected from the SAX vector–producing S3A cells. Infection was for 2 h, a time that had been determined to be adequate to infect CFU-C in vitro (Fig. 2) and to yield a good recovery of viable bone marrow progenitor cells (Table I). The ratios of total infectious SAX virus (as measured by G418-resistant CFU/ml) to total bone marrow cells were 8.3:1 and 4.9:1 in monkeys 56 and 57, respectively. The 14-d CFU-C analysis of an aliquot of the treated marrow cells showed that 25% of the colonies were resistant to 1.7 mg/ml G418 for animal 56 (data not shown) and 28% for animal 57 (Fig. 2, curve *C*). Both primates achieved a rapid and sustained return of normal hematopoietic function with full reconstitution of all blood cell lines entirely analogous to that observed after transplants of untreated autologous bone marrow cells. These animals survived and are healthy more than 1 year after transplantation. Hematopoiesis remains normal

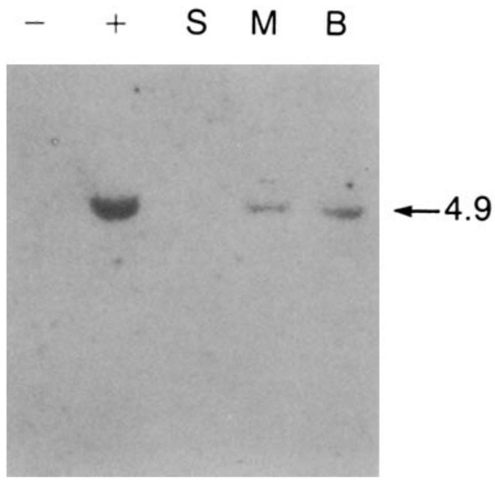

FIGURE 4. Southern blot of DNA extracted from tissues obtained at autopsy on day 69 from animal 10. DNA was prepared from spleen (*S*), bone marrow (*M*), and blood (*B*). Lane *1* (–) contains 30 μg of DNA prepared from peripheral blood mononuclear cells of a control primate. Lane *2* (+) contains 5 pg of the plasmid SAX mixed with 30 μg control primate DNA digested with Kpn I. Lanes *3* (*S*), *4* (*M*), and *5* (*B*) contain 30 μg of DNA prepared from spleen, bone marrow, and blood of animal 10. To determine if the 4.9 kb band in lanes *4* and *5* might be due to contaminating plasmid DNA, the DNA samples were reassayed, but without Kpn I digestion. The band of positive hybridization then appeared at the origin with high-molecular-mass DNA.

in these animals and no evidence of retroviremia has been detected by S^+L^- assay.

Southern blot analyses (of DNA extracted from PBMC obtained on days 34 and 52, as well as of DNA from bone marrow on day 104) for evidence of SAX vector sequences in these two animals were repeatedly negative. This suggests that the vector, if present, was inserted in <5% of cells, that is, below the limits of detection of the method used. PBMC were also analyzed for h-ADA activity and for NPT activity. Both monkeys demonstrated low but readily detectable levels of h-ADA at several times during the posttransplant course (Table I). PBMC from animal 56 contained a level of h-ADA activity equivalent to 0.2% of the endogenous primate activity at day 104; in animal 57, the peak levels of h-ADA on day 69 brought about a 66% conversion of ^{14}C-labelled adenosine to [^{14}C]inosine (Table I), which was calculated to be 0.5% of endogenous primate ADA activity. The separation of human (fractions 2–4) from primate (fractions 16–25) ADA using FPLC and the levels of human and primate ADA activity detected in animal 57 at day 69, expressed as percent adenosine to inosine conversion, are presented in the top panel of Fig. 5. These results are contrasted with those obtained from a control animal that received uninfected autologous bone marrow: this animal (as well as several other control animals) had no detectable activity in the column fractions that would contain h-ADA (Fig. 5, *bottom*) despite greater total protein being loaded onto the column. Sequential assays of PBMC from animals 56 and 57 for h-ADA (Fig. 6) demonstrated h-ADA activity between days 60 and 120. h-ADA was no longer detectable after day 160 in either animal. NPT activity was also detected early in the postransplant period (day 52) but was not detected at day 104 at a time when h-ADA was still discernible (Fig. 7).

In an attempt to determine the frequency of cells expressing the vector-encoded RNA, in situ hybridization was performed using a ^{35}S-labelled *neo*^{r} probe on PBMC obtained from animal 57 on day 127 (Fig. 8). Hybridization was detected in 28 of the 3415 (0.8%) cells counted.

To define the lineage(s) of cells containing the vector, we examined marrow

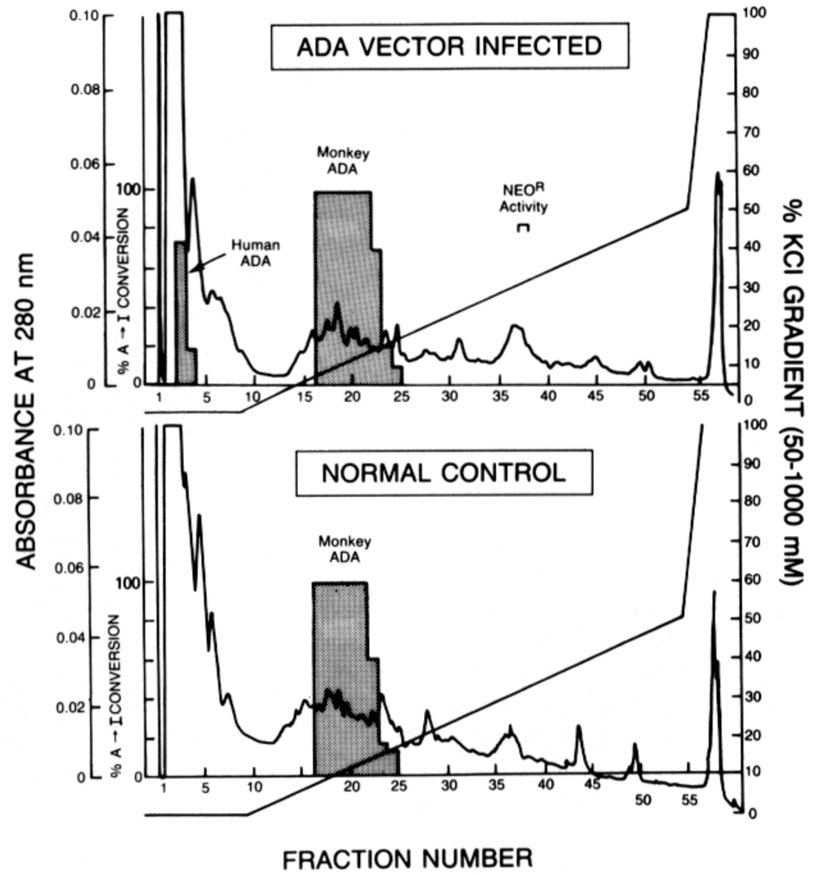

FIGURE 5. FPLC fractionation of hematopoietic mononuclear cell lysates from animal 57 and a noninfected control animal. ADA activity (both endogenous monkey and vector-derived human) in primate 57 bone marrow cells 69 d after transplantation is shown in the upper panel. An uninfected control is shown in the lower panel. The graph represents the absorbance at 280 nm of bone marrow cell lysate fractionated by ion-exchange chromatography on an FPLC Mono Q column using a KCl salt gradient. Stippled bars represent ADA activity expressed as the percent conversion of [^{14}C]adenosine to [^{14}C]inosine (*%A → I*). After thin-layer chromatographic separation of adenosine and inosine, the raw data for fraction 3 (66% conversion) was 9,045 cpm in the [^{14}C]adenosine spot, and 17,889 cpm in the [^{14}C]inosine spot. Background counts were: 407 cpm for [^{14}C]adenosine after 100% conversion (that is, fractions 17–18), and 426 cpm for [^{14}C]inosine after 0% conversion (fraction 1). Fractions containing human ADA, monkey ADA or *neo*^{r} activity are indicated.

CFU-C and clonable peripheral blood T cells for evidence of G418 resistance. Assays of marrow from both animals 57 and 58 for G418-resistant CFU-C performed on days 28, 104, and 169 failed to demonstrate myeloid colonies resistant to the same high concentration (1.7 mg/ml) of G418 that was used to detect CFU-C cultured from vector-infected marrow at the time of transplantation (Fig. 2, Table II). However, with concentrations of G418 lower than maximal (0.29 mg/ml), but still toxic for noninfected controls, resistant colonies were obtained from monkey 56 at day 28 and 169 posttransplant, and from monkey 57 at day 169 posttransplant (Table II).

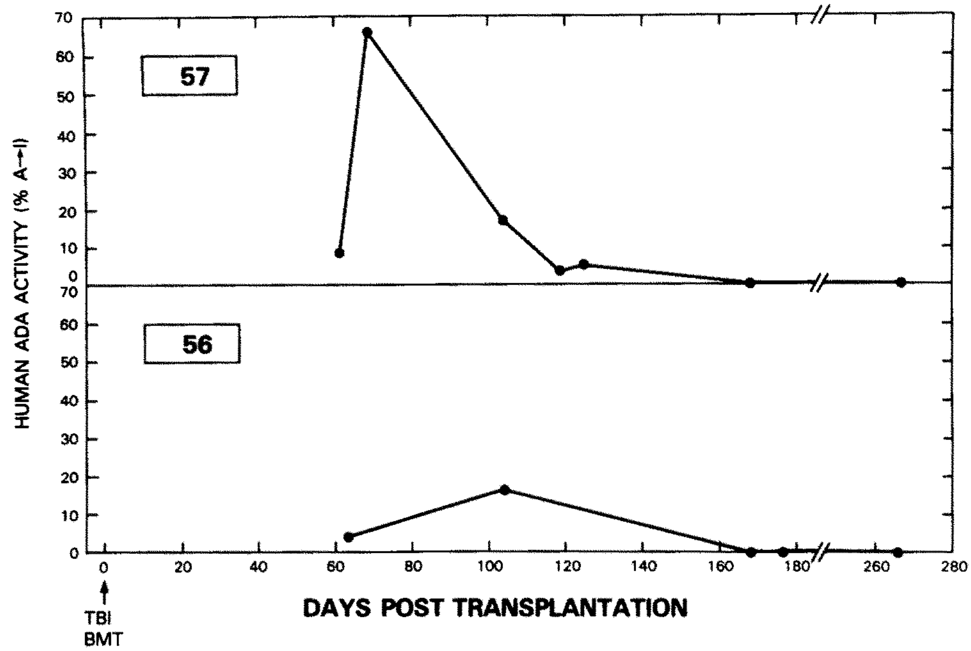

FIGURE 6. The h-ADA activities after FPLC fractionation detected in animals 56 and 57 over time after total body irradiation (*TBI*), retroviral gene transfer, and bone marrow transplantation (*BMT*). The data point at day 69 for animal 57 was taken from Fig. 5, expressed as percent conversion of [^{14}C]adenosine to [^{14}C]inosine.

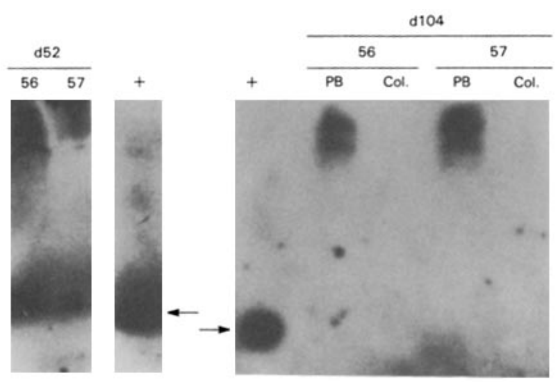

FIGURE 7. The *neo*^{r} phosphotransferase (NPT) activity detected in animals 56 and 57 on days 52 and 104. Bone marrow samples were taken on day 52, and peripheral blood samples were obtained on day 104. The lanes denoted by (+) contained lysate of 5 × 10^5 3T3 cells that had been infected with the N2 vector. The *d52* lanes, denoted by 56 and 57, contained lysate of 10^7 bone marrow cells from day 52. The *d104* lanes denoted as *PB* contained lysates of 10^7 peripheral blood cells and the lanes denoted as *Col.* were the *neo*^{r} fractions from an FPLC Mono Q column that had been used to fractionate 10^8 peripheral blood cells (see Fig. 5). The lanes denoted by (+) and by *d52* 56 and 57 demonstrate the 29 kD band (the size of the NPT enzyme as determined by previous experiments).

In limiting-dilution analyses of peripheral blood lymphocytes, which are used as a means of quantitating the frequency of clonable T lymphocytes expandable in the presence of mitogen (PHA) and IL-2, clonable T cells resistant to concentrations of 0.1 mg/ml G418 were detected in animal 56 at a frequency of 1:93 on day 181 posttransplant (Fig. 9). By comparison, the overall frequency of clonable T cells detected without selection in the PBMC from this animal was one in seven. Thus, ~8% of the clonable T cells detected in the circulation were

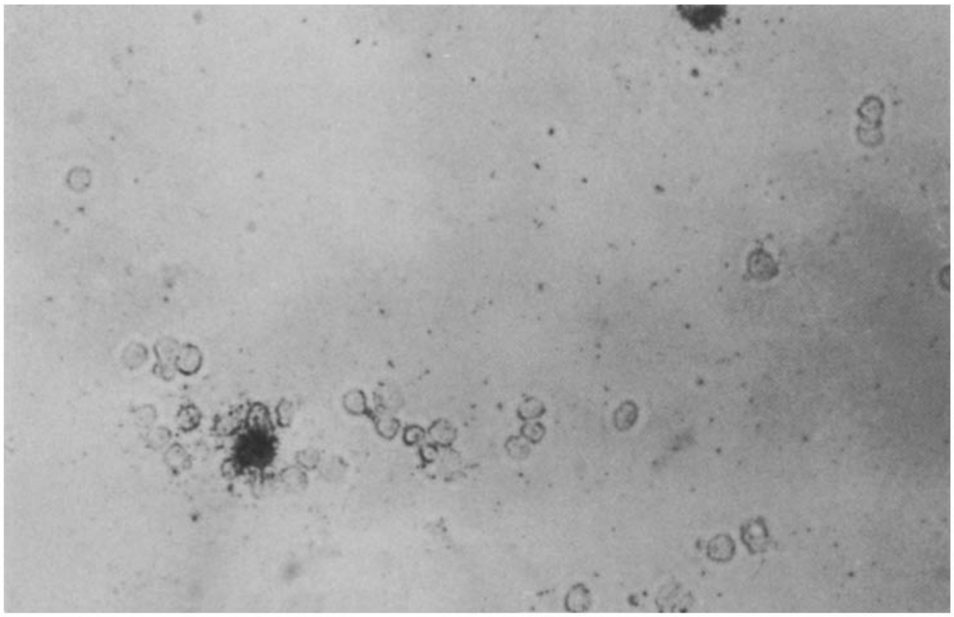

FIGURE 8. In situ hybridization of peripheral blood from animal 57 at day 127. Two positive cells (one, in a cluster of negative cells in the lower left of the figure, and the other at the upper edge on the right) showing overlying silver grains produced by hybridization of an ^{35}S-labelled *neo*^{r} probe to vector-derived mRNA are shown. There were no positive cells found on slides made from uninfected control primate PBMC.

TABLE II

Resistant Bone Marrow Colonies (CFU-C) Assayed at Time of Infection and after Transplantation

Primate marrow cells	G418 (mg/ml)								
	0	0.29	1.71	0	0.29	1.71	0	0.29	1.71
	CFU-C before transplant			CFU-C 28 d after transplant			CFU-C 169 d after transplant		
Uninfected control	37	0	0	26	0	0	55	0	0
Monkey 56	56	17	14	153	5	0	61	1	0
Monkey 57	41	12	12	ND	ND	ND	53	0.6	0

Bone marrow cells were harvested, processed, and incubated with the supernatant from S3A producer cells that generate viral particles (containing the SAX retroviral vector) at a titer of 2×10^6 CFU/ml, as described in the legends of Figs. 2 and 3. After infection, cells were immediately plated in increasing concentrations of G418-containing CFU-C medium. For the posttransplant times, bone marrow mononuclear cells were collected and prepared as described and directly plated into the G418-containing CFU-C medium. The values of colonies are reported for 10^5 cells. The number of cells plated were 3×10^5 for the controls (G418) and 1.2×10^6 for G418-containing points.

resistant to 0.1 mg/ml G418. In contrast, assays of normal cynomolgus monkey lymphocytes have consistently failed to detect clonable T cells resistant to this concentration (0.1 mg/ml) of G418. Further analysis of these G418-resistant clones for h-ADA activity and/or vector DNA sequences could not be performed because of the limited lifetime and number of available cells.

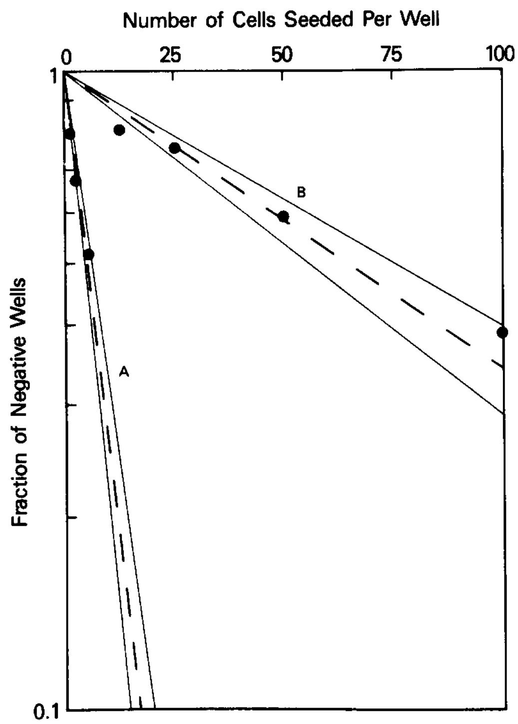

FIGURE 9. Limiting dilution analysis of clonable G418-resistant T cells from peripheral blood mononuclear cells from monkey 56 collected on day 181 after autologous transplantation of SAX-infected marrow. The fraction of negative wells was determined by microscopic examination on day 10 of culture. The slope of line *A* is the frequency (1/7) of clonable T cells in the absence of G418. The slope of line *B* is the frequency (1/93) of clonable T cells in the presence of 0.1 mg/ml of G418. This concentration of G418 completely inhibited growth of T cells isolated from noninfected animals (i.e., no positive wells in four separate control experiments).

The bone marrow cells of two additional animals, 77 and 78, were infected with a modified protocol: the viral supernatant was at pH 7.4 rather than 6.8, and the polybrene was mixed with the supernatant before addition of the marrow cells rather than afterwards. Although both animals reconstituted rapidly (see Fig. 2), animal 77 showed no evidence of h-ADA activity and animal 78 had an h-ADA level equivalent to only 0.05% of endogenous monkey activity (Table 1). In neither case were vector sequences or NPT activity detected. Preliminary analysis using the modified protocol to measure gene transfer into murine CFU-S suggests that both modifications decrease the number of spleen foci that acquire vector DNA sequences (data not shown). Whether these modifications can account for the primate results is still not clear.

Discussion

These studies were undertaken to establish a large animal model to evaluate protocols that might ultimately be used for gene therapy in humans. We have used an amphotropic retroviral vector containing the h-ADA cDNA, SAX, to infect the marrow of cynomolgus primates for subsequent infusion back into donor animals after total body irradiation. Our studies with primates have demonstrated that adequate numbers of autologous bone marrow cells will survive after in vitro culture and exposure to a retroviral vector to provide the animal with full hematopoietic reconstitution and long-term survival. The reconstituted animals have also provided evidence for a low level of successful gene transfer together with expression of the inserted genes.

As detailed in Fig. 2 and Table I, the method of infection of bone marrow cells is an important variable. In previous studies with the mouse, we found that the infection efficiency was 86% for hematopoietic progenitor cells (CFU-S) when these cells were cocultivated with a vector-producing (namely, N2, the parent vector of SAX) cell line (6). Accordingly, we initially used a cocultivation protocol for our early primate transplants. Cocultivation of primate bone marrow cells with the vector-producing cell line for periods of 4–24 h, however, did not achieve as efficient a transfer into marrow progenitors as did incubation with virus-containing supernatants. This was reflected by the consistently lower frequency of G418-resistant CFU-C detected after cocultivation with the producer cell lines. Furthermore, cocultivation with the producer line was associated with bone marrow cell loss, and accordingly with a marked reduction in the total number of bone marrow progenitor cells subsequently available for transplantation. This loss of cells after cocultivation is evident in the transplantation of monkeys 10 and 855, wherein only 15–50% of cells were recovered after cocultivation. This lower recovery of cells undoubtedly contributed to the poor reconstitution of hematopoietic function (particularly in platelet number) achieved in these two monkeys. The four subsequent animals were transplanted with bone marrow cells cultured for 2 h with virus-containing supernatant. Each of these animals achieved full reconstitution of hematopoietic function in the same manner observed in animals receiving autologous, unmanipulated marrow. In vitro studies also indicated that the frequency of G418-resistant colonies transplanted was much higher in the marrow infected with the virus-containing supernatants. Taken together, these data suggest that short-term infection with cell-free virus-containing supernatants provides several advantages for gene transfer experiments in this species. However, to obtain a higher rate of infection, additional modifications of the infection protocol will be necessary.

Our results demonstrate that infection and integration of the retroviral vector SAX in primate marrow cells can be obtained under conditions that favor the recovery of viable cells, and that expression of low levels of the gene products, both h-ADA and NPT enzymatic activities, can be detected for short periods in circulating blood and marrow cells from some reconstituted animals. Although the levels of expression in the PBMC and bone marrow are low, they may represent moderate levels of expression on a per-cell basis. It is unfortunate that, for logistic reasons, we do not have more complete data for animals 56 and 57 during the critical period from 60 to 120 d. The data for animal 57 are summarized in Table III. Because the limit of resolution on our Southern blots is ~1 copy in 20 cells, the negative Southern blots on days 34, 52, and 104 for animal 57 would indicate that vector DNA was present on those days in <5% of the PBMC. The presence of *neo*^{r} RNA in 28 of 3,415 cells as detected by in situ hybridization on day 127 indicates that at that time at least 0.8% of the cells contained vector (more may have had nonexpressing vector DNA sequences). The number of cells containing vector on day 69, when the maximum level of h-ADA was found (0.5% of endogenous monkey ADA activity), is unknown. If 5% of PBMC contained vector on day 69, then infected cells may produce h-ADA at ~10% of endogenous monkey ADA levels (in addition to endogenous monkey ADA already being produced in the cell). If <5% of the cells contained

TABLE III
Summary of Data on Animal 57

Days after transplant	CFU-C*	DNA‡	NPT§	ADA‖	In situ¶	T cells**
0	+++					
34		–				
52		–	+++			
61				+		
69				+++		
104		–	–	++		
119			–	+		
127			–	+	+	
169	±		–	–		
230						+
266			–	–		

* 14-d CFU-C; see Fig. 2 and Table II; the ± on day 169 represents a low percentage of colonies growing at 0.29 mg/ml; see Table II. +++ means >20% G418-resistant CFU-C.
‡ Southern blot analysis.
§ See Fig. 7. +++ means the activity of 10^5–10^6 positive cells.
‖ See Figs. 5 and 6. +++ means >50% conversion of A to I; ++ means 10–50% conversion; + means 1–10%.
¶ See Fig. 8.
** Peripheral blood lymphocytes were cultured that were resistant to 0.25 mg/ml G418.

vector on day 69, then the amount of h-ADA produced on a per-cell basis could exceed 10%.

It should be noted that vectors that do not express genes in one species may be capable of doing so in others. We have used the SAX vector to efficiently transfer the ADA gene into mouse hematopoietic cells but have not detected any ADA expression in vivo in the mouse. We are currently investigating whether this is a vector- and/or promoter-specific phenomenon.

Our studies indicate that the expression obtained appears to be transient, although in both animals, G418-resistant T cells could be obtained as late as 5–7 mo after transplant. The transient expression of vector-derived genes in blood cells obtained from the reconstituted animals could be explained by any of several possibilities. For example, infection might have been restricted to the more differentiated bone marrow cells, which have a limited life span. Alternatively, infection might have been achieved only in a very small number of stem cells, with subsequent progressive dilution of these cells in the absence of a positive selective growth advantage. It is also possible that infected cells undergo cell membrane modification or express extraneous neoantigens that could elicit a response from activated macrophages, NK cells, or immune lymphocytes that are regenerating in the irradiated, marrow-reconstituted host. Finally, the decline in h-ADA activity could be explained by the selective loss of vector sequences due to vector instability, by a decrease in the level of expression of the vector genes in individual cells over time, or by overproduction of ADA, thereby inhibiting or killing positive cells.

Our failure to detect CFU-C equally G418-resistant after transplant as were found before transplant is also consistent with any of the above hypotheses. The detection of G418-resistant clonable T cells in the circulation of animal 56 at day 181 (Fig. 9) and of T cells capable of growth in G418 from animal 57 at day 230 (Table III) also raises the possibility that long-lived T lymphocytes infected with the SAX vector survive for extended periods in these animals.

Vector gene expression among the animals in our series has not been consistent. For example, in monkey 10, h-ADA activity was detected at levels 20–50-fold lower than in monkeys 56 and 57, despite the fact that Southern blot analysis of circulating cells detected vector DNA in this animal. Thus, animal 10 had a higher proportion of cells containing the integrated vector DNA, but expressed the vector at a much lower level. It appears that vector expression in vivo is dependent on variables that are not yet understood. Permanent engraftment of cells containing an expressing gene will probably require the successful infection of pluripotent self-replicating stem cells and a means to maintain positive selective pressure in vivo. Selective pressure may occur for hematopoietic cells containing an expressing ADA gene in patients with ADA deficiency (8).

In conclusion, the data presented indicate that we have been able to obtain h-ADA expression in four out of five reconstituted animals. Four of these animals are still alive >9 mo after transplant. These animals have shown no sign of retroviremia (S^+L^- assays of serum), marrow dysfunction, hematopoietic malignancies, solid tumors, or other signs of pathology. We will continue to study the animals. The S3A cell line produces, in addition to the SAX viral particles, ~0.1% helper virus. Whether or not this low level of helper virus affects the infection frequency is not established (27). It is unlikely, however, that an ongoing productive viral infection was established in vivo, because no evidence of retroviremia has been detected in any of the animals. A complementary gene transfer program using the SAX vector into rhesus monkeys has shown low levels of h-ADA activity (in two out of three animals) and *neo*^{r} activity (in one out of three animals). In addition, these animals have shown no evidence of retroviremia or marrow pathology. Clinical application of this gene transfer protocol will require, we believe, a higher and more stable level of expression of the inserted h-ADA gene as well as greater reproducibility among animals than has been achieved to date.

Summary

Primate bone marrow cells were infected with a retroviral vector carrying the genes for human adenosine deaminase (h-ADA) and bacterial neomycin resistance (*neo*^{r}). The infected cells were infused back into the lethally irradiated donor animals. Several monkeys fully reconstituted and were shown to express the h-ADA and *neo*^{r} genes at low levels in their recirculating hematopoietic cells for short periods of time.

We thank Sheri Bernstein, Jane Selegue, Pat Turner, and Teresa Cartagena for their excellent technical assistance; Brian Agricola, Natalie Antinoff, Ronald Primm, and Kathy Fliszar for the daily care of the animals; Dr. Joseph Pierce for assistance in caring for the animals; and Beth Russo for help with the manuscript.

Received for publication 9 March 1987.

References

1. Joyner, A., G. Keller, R. A. Phillips, and A. Bernstein. 1983. Retrovirus transfer of a bacterial gene into mouse haematopoietic progenitor cells. *Nature (Lond.)*. 305:556.
2. Miller, A. D., D. J. Jolly, T. Friedmann, and I. M. Verma. 1983. A transmissible retrovirus expressing human hypoxanthine phosphoribosyl transferase (HPRT): gene transfer into cells obtained from humans deficient in HPRT. *Proc. Natl. Acad. Sci. USA*. 80:4709.
3. Williams, D. A., I. R. Lemischka, D. G. Nathan, and R. C. Mulligan. 1984. Introduction of new genetic material into pluripotent haematopoietic stem cells of the mouse. *Nature (Lond.)*. 310:476.
4. Dick, J. E., M.-C. Magli, D. Huszar, R. A. Phillips, and A. Bernstein. 1985. Introduction of a selectable gene into primitive stem cells capable of long-term reconstitution of the hemopoietic system of W/W^v mice. *Cell*. 42:71.
5. Keller, G., C. Paige, E. Gilboa, and E. F. Wagner. 1985. Expression of a foreign gene in myeloid and lymphoid cells derived from multipotent haematopoietic precursors. *Nature (Lond.)*. 318:149.
6. Eglitis, M. A., P. W. Kantoff, E. Gilboa, and W. F. Anderson. 1985. Gene expression in mice after high efficiency retroviral-mediated gene transfer. *Science (Wash. DC)*. 230:1395.
7. Hock, R. A., and A. D. Miller. 1986. Retrovirus-mediated transfer and expression of drug resistance genes in human haematopoietic progenitor cells. *Nature (Lond.)*. 320:275.
8. Anderson, W. F. 1984. Prospects for human gene therapy. *Science (Wash. DC)*. 226:401.
9. Parkman, R. 1986. The application of bone marrow transplantation to the treatment of genetic diseases. *Science (Wash. DC)*. 232:1373.
10. Adrian, G. S., D. A. Wiginton, and J. J. Hutton. 1984. Structure of adenosine deaminase mRNAs from normal and adenosine deaminase-deficient human cell lines. *Mol. Cell Biol.* 4:1712.
11. Valerio, D., R. S. McIvor, S. R. Williams, M. G. C. Duyvesteyn, H. van Ormondt, A. J. van der Eb, and D. W. Martin, Jr. 1984. Cloning of human adenosine deaminase cDNA and expression in mouse cells. *Gene*. 31:147.
12. Daddona, P. E., D. S. Shewach, W. N. Kelley, P. Argos, A. F. Markham, and S. H. Orkin. 1984. Human adenosine deaminase. cDNA and complete amino acid sequence. *J. Biol. Chem.* 259:12101.
13. Kantoff, P. W., D. B. Kohn, H. Mitsuya, D. Armentano, M. Sieberg, J. A. Zwiebel, M. A. Eglitis, J. R. McLachlin, D. A. Wiginton, J. J. Hutton, S. D. Horowitz, E. Gilboa, R. M. Blaese, and W. F. Anderson. 1986. Correction of adenosine deaminase deficiency in cultured human T and B cells by retrovirus-mediated gene transfer. *Proc. Natl. Acad. Sci. USA*. 83:6563.
14. Williams, D., S. H. Orkin, and R. C. Mulligan. 1986. Retrovirus-mediated transfer of human adenosine deaminase gene sequences into cells in culture and into murine hematopoietic cells in vivo. *Proc. Natl. Acad. Sci. USA*. 83:2566.
15. Belmont, J. W., J. Henkel-Tigges, S. M. Chang, K. Wager-Smith, R. E. Kellems, J. E. Dick, M.-C. Magli, R. A. Phillips, A. Bernstein, and C. T. Caskey. 1986. Expression of human adenosine deaminase in murine haematopoietic progenitor cells following retroviral transfer. *Nature (Lond.)*. 322:385.
16. Armentano, D., S.-F. Yu, P. W. Kantoff, T. von Ruden, W. F. Anderson, and E. Gilboa. Effect of internal viral sequences on the utility of retroviral vectors. *J. Virol.* 61:1647.

17. Pike, B. L., and W. A. Robinson. 1970. Human bone marrow colony growth in Agar-gel. *J. Cell Physiol.* 76:77.
18. Gregory, C. J., and A. C. Eaves. 1977. Human marrow cells capable of erythropoietic differentiation in vitro: Definition of three erythroid colony responses. *Blood.* 49:855.
19. Gross-Bellard, M., P. Oudet, and P. Chambon. 1973. Isolation of high molecular weight DNA from mammalian cells. *Eur. J. Biochem.* 36:32.
20. Southern, E. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503.
21. McLachlin, J. R., S. C. Bernstein, and W. F. Anderson. 1986. Separation of human from mouse and monkey adenosine deaminase by ion exchange chromatography following retroviral-mediated gene transfer. *Anal. Biochem.* In press.
22. Soberman, R. J., and M. L. Karnovsky. 1980. Metabolism of purines in macrophages. Effect of functional state of the cells. *J. Exp. Med.* 152:241.
23. Reiss, B., R. Sprengel, H. Will, and H. Schaller. 1984. A new sensitive method for qualitative and quantitative assay of neomycin phosphotransferase in crude cell extracts. *Gene.* 30:211.
24. Singer, R. H., J. B. Lawrence, and C. Villnave. 1986. Optimization of in situ hybridization using isotopic and non-isotopic detection methods. *Biotechniques.* 4:230.
25. Kernan, N. A., N. Flomenberg, N. H. Collins, R. J. O'Reilly, and B. Dupont. 1985. Quantitation of T lymphocytes in human bone marrow by a limiting dilution assay. *Transplantation (Baltimore).* 40:317.
26. Miller, A. D., R. J. Eckner, D. J. Jolly, T. Friedmann, and I. M. Verma. 1983. Expression of a retrovirus encoding human HPRT in mice. *Science (Wash. DC).* 225:630.
27. Hogge, D. E., and R. K. Humphries. 1987. Gene transfer to primary normal and malignant human hemopoietic progenitors using recombinant retroviruses. *Blood.* 69:611.